REMARKS

I. In item 2 of the Office Action, claims 1-3, 8, 27, 28, 30, 31, 43 and 45-47 were rejected under 35 U.S.C. §102(b) over WO 99/26480.

The rejection is traversed for the following reasons.

WO 99/26480 does not provide a written description of, and does not enable direct administration of an endostatin-expressing vector to the eye to obtain an antiangiogenic effect as claimed in the instant application.

No particular teaching of how to deliver endostatin to the eye using a vector is provided in WO 99/26480. The working examples relate to angiostatin or an angiostatin fusion, and are directed to treating cancer. There is no teaching of transferring endostatin alone for a non-cancer treatment of the eye. Moreover, in light of the lack of evidence of endostatin use in treating cancer, WO 99/26480 is not enable for treating ocular cancer using endostatin as well.

WO 99/26480 has not matured to patent. There is every indication the PCT application was not pursued and is abandoned. Attached hereto are printouts (3 pages) from a database of international patent documents. There are only two applications in the family, the PCT application and a corresponding Australia application, first page of printout. There is no prosecution activity in the Australia application, second page of printout. On the third page, it can be seen that no pending applications matured from the PCT application.

Without evidence of maturing to patent, and with evidence that the application was abandoned, WO 99/26480 does not carry a presumption of enablement as does an issued patent. Instead, as applications often are filed early in development, enablement and written description can be questionable, and particularly in unpredictable arts.

WO 99/26480 is not an enabled reference, an artisan would recognize such, and thus, is not an effective reference for teaching the use of endostatin as an antiangiogenic in the eye as described and claimed in the instant application.

There are several additional reasons to support the conclusion that WO 99/26480 is not enabled and thus not an effective reference.

(1) The Nature Of The Invention – It Is Unknown Whether Endostatin Is Anti-Angiogenic Under All Circumstances And In All Conditions.

In 1998, it was clear much of the original research on endostatin could not be repeated, programs were terminated and clinical trials were unsuccessful, see the copy of King attached to the Declaration of Connelly, and discussed therein beginning at the bottom of page 1 and including the first two full paragraphs on page 2. NCI scientists were unable to demonstrate an antiangiogenic activity, page 1, fourth full paragraph of King. In that same paragraph, King reported that Genentech tried to duplicate the research over a period of a year without success and terminated their program. EntreMed, the Maryland company that licensed to commercialize endostatin, could not duplicate results, page 1, fifth full paragraph of King.

In 2002, EntreMed reported in their 3Q SEC report that they would not initiate new clinical trials because of equivocal results.

Thus, WO 99/26480 relates to a molecule of uncertain biological activity for the treatment of cancer. That speaks to a molecule that is not enabled because of a lack of reproducibility. There is unpredictability in the art of endostatin. It follows then, that WO 99/26480 is not enabled for antiangiogenesis in non-cancer settings, and in tissues other than those with cancer.

(2) The State of the Art – Angiogenesis Is A Complex Process Within and Between Tissues.

Eberhard et al. (Canc. Res. 60, 1388, 2000, copy attached hereto) teach that angiogenesis patterns vary within a tumor and between different kinds of tumors, Abstract, last two sentences. Eberhard et al. also found that angiogenesis patterns of tumors differ from that of normal tissue, ovarian corpus rubrum.

The variability in angiogenesis within a tissue and between tissues suggests complexity of angiogenesis, and that different mechanisms may exist for different tissues and at different times during the growth of a single tissue.

As further evidence of the variability of angiogenesis, Berger et al. (J. Surg. Res. 91(1), 26, 2000, copy attached hereto) observed that endostatin had no effect in wound healing. Subsequent studies revealed that endostatin has no effect on angiogenesis in pregnancy and in tissue repair.

Thus, angiogenesis is complex and variable. In light of that knowledge, it is clear WO 99/26480 had no appreciation for the tissue variability and the temporal variability within a

as there is no particularized teachings of angiogenesis in the various tissues. Without such treatment, it is unpredictable whether endostatin would be operable in any one tissue, such as the eye.

As discussed above, below and in the record, endostatin is inoperable, without antiangiogenesis activity, in several models and tissues characterized by angiogenesis. The unpredictability of endostatin is imposed on the variability of angiogenesis from tissue to tissue. WO 99/26480 does not address those factors.

(3) The Art Is Unpredictable – The WO 99/26480 Inventor Cannot Duplicate The Teachings Of WO 99/26480.

Jouanneau et al. (copy attached to the Declaration of Connelly, and discussed in the paragraph bridging pages 3 and 4 thereof) reported a lack of antitumor activity of endostatin in a tumor model. One of the co-authors is Philippe Leboulch, the named first inventor of WO 99/26480. In Jouanneau et al., the SKNAS cell line was injected into nude mice, similar to the method taught in Example 5 of WO 99/26480. Although serum endostatin levels were high, no alteration in tumor growth was noted.

In the article of Mandavilli dated 18 March 2002, copy attached to the Declaration of Connelly and discussed on page 3 of the Declaration, two papers reporting an inability to demonstrate an antiangiogenic activity for endostatin were summarized. Both of those papers were co-authored by Dr. Leboulch, a named inventor of WO 99/26480. Dr. Leboulch was quoted in Mandavilli, "We could not see an effect of endostatin any way we tried."

Eisterer et al. (attached to the Declaration of Connelly and discussed on page 4 thereof) is one of the articles referred to by Mandavilli. Leboulch is a co-author of the publication. Hematopoietic stem cells were transformed to express endostatin. Despite substantial serum levels of endostatin after transplantation into mice, growth of leukemia cells in the engrafted mice was not altered.

Since Leboulch reported not being able to demonstrate an antiangiogenic activity of endostatin, coupled with the other negative reports of endostatin, an artisan would conclude that there is a lack of predictability of endostatin and the biological activities attributed thereto. An invitation to experiment is not a proper basis to ground an obviousness rejection, and certainly, not in an unpredictable art where it would require undue experimentation to replicate the

teachings. As the inventor of WO 99/26480 could not replicate the teachings therein, the inescapable conclusion is that WO 99/26480 is not enabled and does not describe the subject matter of interest.

(4) The Amount of Direction In WO 99/26480 Is Insufficient.

As argued in the record, WO 99/26480 provides a generic teaching of endostatin. The protein or gene is delivered by any means, using any form of endostatin, to any cell to treat any angiogenesis-related disease. There are factors that need to be considered in gene therapy, for example, whether a vector can express endostatin, and in suitable levels, whether the endostatin is biologically active, whether the endostatin is sited in proximity to cells in need of endostatin, simply, the overall bioavailability to the cells in need of endostatin. As discussed hereinabove, angiogenesis is a complex and variable process. WO 99/26480 does not discuss the details of ocular gene therapy, for example, whether a vector can operate in the eye, whether endostatin can be expressed in the eye, whether endostatin can be biologically active with target cells in the eye and so on.

When Leboulch of WO 99/26480 repeatedly reported that he was unable to practice the several methods taught in WO 99/26480, and with the very superficial mention of a wide variety of materials and methods for practicing the teachings in WO 99/26480, there is but the inescapable conclusion that WO 99/26480, in the unpredictable arts of angiogenesis and endostatin, is wholly insufficient in providing an artisan with an enabled written description for treating ocular disorders typified by angiogenesis using endostatin.

In light of the state of the art discussed hereinabove and in the record, the complexity of angiogenesis processes and the numerous reports on the lack of endostatin activity in certain types of cells, an artisan would realize that additional direction is needed for treating ocular disorders presenting with angiogenesis using endostatin.

(5) There Are No Endostatin Working Examples In WO 99/26480.

Of the eleven working examples in WO 99/26480, seven are prophetic.

Example 4 demonstrated detectable expression from retroviral vectors of angiostatin constructs, not endostatin, page 2, lines 14-16.

Example 5 demonstrated an alleged in vivo impairment of tumor growth by the angiostatin-endostatin fusion protein of Example 2. However, there are five different

angiostatin-endostatin fusion proteins disclosed in Example 2 and it is unknown which was used in the studies reported in Example 5. Nevertheless, at the least, use of endostatin alone is not described.

Thus, there are no working examples demonstrating the successful expression and use of endostatin to treat any model of angiogenesis.

Examples 6-11 are prophetic examples that describe an in vivo model similar to that described in Eisterer et al. discussed hereinabove, which demonstrated no antiangiogenic activity.

Accordingly, there is no evidence provided in WO 99/26480 that endostatin is antiangiogenic. In fact, there is no reduction to practice, just a wish to express endostatin and just a wish to use endostatin as an antiangiogenic agent in any particular cell or tissue. At best, that is but an invitation to experiment, which is not a basis on which to assert obviousness.

Certainly there is no working example of using endostatin in the eye to obtain antiangiogenesis activity in the eye. That notion of making and using endostatin in WO 99/26480 is inoperable. Thus, in the area of patents, is not a proper conception and not an invention; and in the area of science, is not reproducible and therefore, not real.

(6) WO 99/26480 Does Not Teach Or Describe Use Of Endostatin In The Eye.

WO 99/26480 does not provide any details on the making and using of endostatin to treat ocular diseases. WO 99/26480 does not teach or describe any particularities of angiogenesis in the eye.

Because no experimentation to make and to use endostatin to treat ocular diseases is provided in WO 99/26480, an undue amount of experimentation is needed to practice the teachings of WO 99/26480 for reducing angiogenesis in ocular diseases with a reasonable expectation of success, particularly in light of the state of the art.

For the reasons above and of record, WO 99/26480 does not satisfy the written description and enablement requirements of a reference as to treating ocular diseases. Thus, WO 99/26480 is not an effective reference as to the invention of interest.

Perhaps most probative on concluding that WO 99/26480 is not an effective reference is the observation that Dr. Phillipe Leboulch, named as an inventor on WO 99/26480 and

recognized leader in the study of endostatin, was unable to reproduce the teachings of WO 99/26480. He conducted several studies aimed at exposing whether endostatin had any in vivo antiangiogenic activity in treating tumors. In a move generally atypical for science, Leboulch published negative data demonstrating that in a number of models for treating cancer, endostatin demonstrated no antiangiogenic activity.

One example of such a report that demonstrates the non-enablement of WO 99/26480 is Pawliuk et al., Mol. Therap. 5, 345, 2002, copy attached hereto. Leboulch is the senior author of the paper. Hematopoietic stem cells were transformed with a retrovirus encoding endostatin. Despite continuous, high level expression of endostatin, there was no inhibition of neoangiogenesis and no antitumor activity in vivo. Thus, Leboulch was unable to demonstrate any antiangiogenesis activity for endostatin in vivo.

If a recognized expert in endostatin cannot demonstrate antiangiogenesis activity for endostatin in cancer, it is not expected that one of ordinary skill in the art could do so. Clearly, then, one must conclude that WO 99/26480 is not enabled, the named inventor could not reproduce the teachings therein.

As WO 99/26480 is not an effective reference as to the instant invention, WO 99/26480 does not place the claimed invention relating to treating ocular disorders enably in the hands of the artisan. Hence, there is no anticipation. Accordingly, withdrawal of the rejection is requested respectfully.

II. On pages 3-9 of the Office Action, claims 1 and 29; claims 1 and 32; claims 1, 33 and 38; and claims 1, 33, 38-41 and 48-50 were rejected under 35 U.S.C. §103(a) over four combinations of references. In each of the four rejections, WO 99/26480 is the primary reference. The Examiner detailed a number of deficiencies in WO 99/26480 in constructing the four rejections.

The four rejections are traversed for the following reasons.

As discussed in Section I hereinabove, and in the record, and herein incorporated by reference, WO 99/26480 is not enabled as to the claimed invention and thus is legally insufficient to teach or to suggest ocular gene therapy using endostatin.

For that reason alone, because the primary reference is legally insufficient for all four rejections, prima facie cases of obviousness have not been made.

None of the secondary or tertiary references cures the fatal deficiencies of WO 99/26480. Therefore, a prima facie case of obviousness was not made in any of the four rejections.

For example, Keshet et al. do not relate to ocular disorders. Keshet et al. relate to cancer. As provided in the record, angiogenesis is a very diverse process from tissue to tissue and from cell to cell. Thus, absent evidence of a correlation, there is no basis to conclude that the stimulation or inhibition of angiogenesis by a factor in one disorder, in one tissue or in one cell will be operable in another disorder, another tissue or another cell. Accordingly, Keshet et al. do not cure the deficiencies of the primary reference. Keshet et al. do not enable use of endostatin for ocular gene therapy.

Otani et al. do not relate to gene therapy and do not relate to cancer. Therefore, Otani et al. is from non-analogous art as to WO 99/26480 or Keshet et al. Otani et al. do not cure or enable WO 99/26480.

In all of the four rejections of alleged obviousness, the issue is whether an artisan would have a reasonable expectation of successfully obtaining ocular therapy using endostatin. The evidence of record and the two Declarations attached hereto demonstrate that an artisan would not have a reasonable expectation of success because the primary reference does not enable and thus does not teach or describe use of endostatin in a vector to treat ocular neovascularization. Angiogenesis, and the inhibition thereof, in cancer and in non-cancer conditions is diverse, and the factors that stimulate and inhibit angiogenesis are diverse.

Attached hereto is the Declaration of Connelly. In the Declaration, Dr. Connelly confirms the knowledge, not always published, of negative results on endostatin activity, or lack thereof. Aside from the exhibits discussed hereinabove, Dr. Connelly discusses a brief paragraph appearing in Science magazine in the third full paragraph of page 3; and on page 4 discussed the publication of Bachelot et al., yet another article co-authored by Leboulch reporting no antiangiogenesis activity for endostatin. Endostatin research was not reproducible, not consistent and not practicable. Dr. Connelly refers to several publications and reports attesting to the state of endostatin research, namely the lack of reproducibility of the original research and the growing skepticism that endostatin has any antiangiogenesis activity, particularly in cancer,

using a variety of models. Dr. Connelly observed that endostatin experiments relating to delivery of the endostatin gene as compared to delivery of endostatin protein yielded more fluctuating and disappointing results.

Dr. Connelly also provides a conclusion by one of ordinary skill in the art as to what is obtained on reading WO 99/26840, whether alone or in light of the state of the art at the time the instant application was filed. Because it was unknown whether endostatin was antiangiogenic and because of the variability in angiogenesis in various tissues, she concluded that WO 99/26840 is insufficient in providing a reproducible protocol (that is, non-enabling without a clear description) for using endostatin, and particularly in the eye, as there is but passing mention of the eye in a "laundry list" of tissues, without any disclosure on the nature of angiogenesis in the eye, and whether endostatin would have any such antiangiogenesis activity in the eye. That confusion, lack of reproducibility and complexity in the arts of endostatin and angiogenesis, exist to this day.

Moreover, it should be noted that publications by a named inventor of WO 99/26480 stating that he could not reproduce the teachings in WO 99/26480 or obtain any evidence that endostatin has antiangiogenic activity demonstrate the non-enablement of WO 99/26480. Endostatin is an unpredictable art and it would require undue experimentation to practice endostatin with a reasonable expectation of success. That is clear evidence that WO 99/26480 is not a legally sufficient reference for using endostatin in ocular disorders.

Also attached hereto is the Declaration of Kaleko.[1] Dr. Kaleko describes how the research disclosed in the instant application was proposed and the surprise generated with the unexpected observation of endostatin antiangiogenic activity in the eye. The use of endostatin was not based on an expectation that an antiangiogenic activity was going to be obtained, instead, endostatin was used essentially as a transgene reporter to validate the vector for use in the eye and to begin the research to prove the program was worthy of funding, particularly when the preferred transgene of interest, not endostatin, was obtained and tested.

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[1] Attached hereto are Petition To Correct Inventorship documents relating to adding Drs. Kaleko and Luo as co-inventors. The fully executed Declaration/Power of Attorney will be filed as soon as possible.

That unexpected result, in light of the unpredictable state of the art as to endostatin, and particularly in treating ocular diseases, speaks to the nonobviousness of the instant invention.

Accordingly, prima facie cases of obviousness have not been made. There is no enablement in the primary reference, WO 99/26480, and no reasonable expectation of success in the use of endostatin, in general, and in particular, in the eye. The unexpected observation of antiangiogenic activity of endostatin in the eye despite the skepticism in the state of the art, overcomes any prima facie case of obviousness. Hence, withdrawal of the four §103(a) rejections is requested respectfully.

CONCLUSION

Applicants submit that the pending claims are in condition for allowance and early indication of such is requested respectfully. Reexamination, reconsideration, withdrawal of the rejections and early passage of the application to issuance are solicited earnestly. If any fees are found to be applicable, please charge any additional fees or make any credits to Deposit Account No. 02-1818.

Respectfully submitted,

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Dated: 8 February 2006

Family list
2 family members for:
WO9926480
Derived from 2 applications.

Back to WO9926480

1 **Anti-angiogenic gene therapy vectors and their use in treating angiogenesis-related diseases**
Publication info: AU1598599 A - 1999-06-15

2 **ANTI-ANGIOGENIC GENE THERAPY VECTORS AND THEIR USE IN TREATING ANGIOGENESIS-RELATED DISEASES**
Publication info: WO9926480 A1 - 1999-06-03

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Anti-angiogenic gene therapy vectors and their use in treating angiogenesis-related diseases

Legal status (INPADOC) of AU1598599

No legal data found.

ANTI-ANGIOGENIC GENE THERAPY VECTORS AND THEIR USE IN TREATING ANGIOGENESIS-RELATED DISEASES

Legal status (INPADOC) of WO9926480

WO F	9824950 W (Patent of invention)
PRS Date :	1999/06/03
PRS Code :	AK
Code Expl.:	+ DESIGNATED STATES
KD OF CORRESP. PAT.:	A1
DESIGNATED COUNTR.:	AL AM AT AU AZ BA BB BG BR BY CA CH CN CU CZ DE DK EE ES FI GB GE GH GM HR HU ID IL IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT UA UG UZ VN YU ZW
PRS Date :	1999/06/03
PRS Code :	AL
Code Expl.:	+ DESIGNATED COUNTRIES FOR REGIONAL PATENTS
KD OF CORRESP. PAT.:	A1
DESIGNATED COUNTR.:	GH GM KE LS MW SD SZ UG ZW AM AZ BY KG KZ MD RU TJ TM AT BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG
PRS Date :	1999/08/18
PRS Code :	121
Code Expl.:	EP: THE EPO HAS BEEN INFORMED BY WIPO THAT EP WAS DESIGNATED IN THIS APPLICATION
PRS Date :	1999/08/19
PRS Code :	DFPE
Code Expl.:	REQUEST FOR PRELIMINARY EXAMINATION FILED PRIOR TO EXPIRATION OF 19TH MONTH FROM PRIORITY DATE
PRS Date :	2000/05/20
PRS Code :	NENP KR
Code Expl.:	NON-ENTRY INTO THE NATIONAL PHASE IN:
PRS Date :	2000/09/21
PRS Code :	REG DE 8642
Code Expl.:	- IMPACT ABOLISHED FOR DE - I.E. PCT APPL. NOT ENT. GERMAN PHASE
PRS Date :	2001/03/07
PRS Code :	122
Code Expl.:	- EP: PCT APP. NOT ENT. EUROP. PHASE
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Code Expl.:	NON-ENTRY INTO THE NATIONAL PHASE IN:

[CANCER RESEARCH 60, 1388–1393, March 1, 2000]

Heterogeneity of Angiogenesis and Blood Vessel Maturation in Human Tumors: Implications for Antiangiogenic Tumor Therapies[1]

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ABSTRACT

Microvessel density (MVD) counting techniques have been widely used to assess the vasculature in tumors. MVD counts assess the presence of blood vessels but do not give an indication of the degree of angiogenesis and the functional status of the tumor neovasculature. To analyze angiogenesis and the functional status of the tumor vascular bed, we have quantitated endothelial cell proliferation and the recruitment of pericytes in human tumors [glioblastomas (n = 30), renal cell carcinomas (n = 22), colon carcinomas (n = 18), mammary carcinomas (n = 24), lung carcinomas (n = 15), and prostate carcinomas (n = 19)]. These findings were compared to the physiological angiogenesis in the cyclic bovine ovarian corpus luteum. Tissue sections were examined applying double-labeling immunohistochemical techniques to detect proliferating endothelial cells and to colocalize endothelial cells and pericytes. The following parameters were quantitated: (*a*) MVD count; (*b*) proliferating capillary index (PCI); (*c*) proliferating tumor *versus* endothelial cell index; and (*d*) microvessel pericyte coverage index (MPI). Based on endothelial cell proliferation, angiogenesis was found to be present in all tumors with characteristic and significant differences between the tumor types (glioblastomas, PCI = 9.6 ± 6.1%; renal cell carcinomas, PCI = 9.4 ± 5.2%; colon carcinomas, PCI = 7.8 ± 5.2%; mammary carcinomas, PCI = 5.0 ± 4.8%; lung carcinomas, PCI = 2.6 ± 2.5%; prostate carcinomas, PCI = 2.0 ± 1.4%). There was a considerable degree of heterogeneity in the intensity of angiogenesis within each tumor group, as indicated by large standard deviations. Even in the most angiogenic tumors, angiogenesis was found to be 4 to 20 times less intense as compared with the physiological angiogenesis in the growing ovarian corpus rubrum (PCI = 40.6 ± 6.2%). Varying degrees of pericyte recruitment to the tumor microvasculature were determined in the different tumor types (glioblastomas, MPI = 12.7 ± 7.9%; renal cell carcinomas, MPI = 17.9 ± 7.8%; colon carcinomas, MPI = 65.4 ± 10.5%; mammary carcinomas, MPI = 67.3 ± 14.2%; lung carcinomas, MPI = 40.8 ± 14.5%; prostate carcinomas, MPI = 29.6 ± 9.5%). The data demonstrate distinct quantitative variations in the intensity of angiogenesis in malignant human tumors. Furthermore, the varying degrees of pericyte recruitment indicate differences in the functional status of the tumor vasculature in different tumors that may reflect varying degrees of maturation of the tumor vascular bed.

INTRODUCTION

Tumor growth and metastatic dissemination are critically dependent on the tumor's supply of blood vessels (1–3). The angiogenesis dependency of tumor growth has led to the development of antiangiogenic therapies that are conceptually extremely appealing for a number of reasons (4–6): (*a*) as an oncofetal mechanism that is mostly down-regulated in the healthy adult, targeting of angiogenesis should lead to minimal side effects even after prolonged treatment; (*b*) tumor-associated angiogenesis is a physiological host mechanism; consequently, its pharmacological inhibition should not lead to the development of resistance (7); (*c*) each tumor capillary potentially supplies hundreds of tumor cells, and the targeting of the tumor vasculature should thus lead to a potentiation of the antitumorigenic effect: and (*d*) in contrast to the interstitial location of tumor cells, direct contact between the vasculature and the circulation allows efficient access to therapeutic agents.

Despite the enormous efforts aimed at elucidating the molecular determinants of angiogenesis (8–10) and the intense search for natural and synthetic angiogenesis inhibitors (4, 6), surprisingly little is known about the nature of the vascular bed in human tumors. Almost all of the studies that have assessed endothelial cell turnover in tumors were performed in experimental animal models with rapidly growing tumors whose growth kinetics are vastly different from the growth kinetics of human tumors (11, 12). In fact, the few endothelial cell turnover studies that have been performed in human tumors do suggest that endothelial cell proliferation in these tumors is detectable, albeit at a relatively low rate (13–16). Average tumor endothelial cell proliferation indices of 0.15% have been reported for prostatic carcinomas (13). The endothelial cell labeling index in mammary carcinoma varies between 2.2% (14) and 2.7% (15), and a value as high as 9.9% has been reported for colorectal adenocarcinomas (16).

As early as 1972, Brem *et al.* (17) proposed a microscopic angiogenesis grading system to assess the angiogenic status of the tumor vasculature. Based on the analysis of the vascular density, the number of endothelial cell nuclei, and the cytological properties of tumor-associated endothelial cells, an angiogenesis score was determined and used to establish an angiogenic rank order of different human brain tumors (17). In recent years, the vascular bed of human tumors has been characterized extensively by performing MVD[4] counting studies (18, 19). These studies have revealed that high MVD counts within vascular hot spots of tumors correspond with a poor prognosis for the patient. MVD studies using panendothelial cell markers reflect the vascular status of a tissue, *i.e.*, the presence of blood vessels. However, they do not give an indication of the angiogenic status of a tissue vascular bed, *i.e.*, the rate of ongoing angiogenesis and the functional status of tumor neovasculature. To more realistically assess the angiogenic status of the vasculature within human tumors, the present study was aimed at functionally analyzing the properties of the tumor vascular bed. Based on the analysis of tumor endothelial cell proliferation and pericyte recruitment, angiogenesis and the functional status of the tumor microvascular bed were quantitated in six different types of malignant human tumors. These findings were compared with the angiogenesis kinetics in the cyclic ovarian corpus luteum, one of the few organ sites in the adult with significant physiological angiogenesis.

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[2] A. E. and S. K. contributed equally to this work.

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[4] The abbreviations used are: MVD, microvessel density; PCI, proliferating capillary index; PTE, proliferating tumor *versus* endothelial cell; MPI, microvessel pericyte coverage index; α-SMA, α-smooth muscle actin; vWF, von Willebrand factor; PCNA, proliferating cell nuclear antigen; BS-I, *Bandeiraea simplicifolia* I; AEC, amino ethyl carbazole; HPF, high power field.

MATERIALS AND METHODS

Tissue Samples. Tissue specimens of formalin-fixed paraffin-embedded human tumors were retrieved from the pathology archive. All tumors (1–4 blocks/tumor) were histopathologically evaluated by two investigators independently of the original pathology report. Tumor-free tissue adjacent to the tumor as well as tumor-free specimen from necropsies served as normal control tissue. Specimens of bovine ovaries were retrieved from an established bank of tissues that has been extensively analyzed for its angiogenesis status (20, 21).

Staining of Proliferating Endothelial Cells. A double-labeling immunohistochemical technique was used to simultaneously stain nuclei of proliferating cells and endothelial cells. Deparaffinized and rehydrated sections (4 μm) were microwaved, endogenous peroxidase was blocked, and sections were incubated with an antibody to PCNA (clone PC10; 1:100 dilution; final concentration, 3.9 μg/ml; Dako, Hamburg, Germany; 60 min, room temperature) or Ki67 (clone MIB1; 1:10 dilution; final concentration, 20 μg/ml; Dianova, Hamburg, Germany; 60 min, room temperature). A biotinylated secondary antibody, streptavidin alkaline phosphatase complex, and nitroblue tetrazolium as a substrate (Zymed, South San Francisco, CA) were used to visualize binding of the first antibody. Single-color-stained tissue sections were incubated with double-staining enhancer (Zymed) for 30 min, and then endothelial cells were stained for CD34 expression (human tissues: clone QBEnd/10; 1:25 dilution; Novocastra, Newcastle, United Kingdom; 2 h, room temperature; secondary antibody; Zymed) or binding of the lectin BS-I (bovine tissues; biotinylated BS-I; 10 μg/ml; Sigma, Deisenhofen, Germany; 37°C, 2 h) using streptavidin-peroxidase as enzyme and AEC as chromogenic substrate (Zymed).

Staining of Mural Cells. To quantitatively assess the pericyte coverage of microvessels, a double-labeling immunohistochemical technique was used to simultaneously stain endothelial cells (CD34 or vWF) and mural cells (α-SMA). Of the analyzed tumors, 25% of archive-retrieved specimens were not suitable for the CD34/α-SMA double-staining technique. Deparaffinized and rehydrated tissue sections were peroxidase-blocked, trypsinized, incubated with blocking serum, and then double-stained for α-SMA expression to detect pericytes and smooth muscle cells, followed by CD34 staining (human tumors) or vWF staining (bovine ovaries) to label endothelial cells. For α-SMA staining, sections were incubated with a monoclonal mouse antihuman α-SMA antibody (clone 1A4; 1:400 dilution; final concentration, 20 μg/ml; Sigma) for 2 h at room temperature. A biotinylated secondary antibody, streptavidin alkaline phosphatase complex, and nitroblue tetrazolium as substrate (Zymed) were used to visualize binding of the α-SMA antibody. Subsequent staining of endothelial cells was essentially performed as described above using an antibody to CD34 to stain endothelial cells in human tumors and a polyclonal antiserum to vWF (polyclonal rabbit antihuman vWF antiserum; 1:200 dilution; final concentration, 28.5 μg/ml; DAKO).

Quantitation of MVDs, PCI, and MPI. Sections were assessed for uniformity of staining at low power (×100), and individual microvessel counts were then performed in on a ×400 field. To express MVD counts microscope-independent, counts were transformed and expressed as the number of microvessels/mm² (1 HPF = 0.0681 mm²). Density counts of CD34-, BS-I-, or vWF-stained microvessels were performed independently by three investigators, as described previously (18, 20). At least five independent microscopic fields per tissue section were analyzed by two independent investigators to count PCNA-positive tumor cells and endothelial cells. Tumor cell proliferation and endothelial cell proliferation were quantitated in vascular hot spots that were identified by screening for the areas with highest vessel density at low magnification. A PCI was determined by calculating the ratio of the number of microvessels with proliferating endothelial cells:the total number of microvessels. A MPI was correspondingly established by quantitating the percentage of microvessels that colocalized endothelial cell staining (CD34 or BS-I) and pericyte staining (α-SMA). For MPI quantitation, at least five independent microscopic fields per section were independently analyzed by two investigators.

Statistical Analysis. Results were analyzed for statistical significance by an ANOVA and the Mann-Whitney *U* test. Two-sided statistical calculations were performed using the Statistica 5.1 program (StatSoft, Tulsa, OK) on an IBM-compatible personal computer.

RESULTS

Endothelial Cell Proliferation in Human Tumors. Angiogenesis and pericyte recruitment were assessed in six different types of human tumors that were all histologically diagnosed as malignant tumors, namely, glioblastomas, renal cell carcinomas, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas (Table 1). To quantitate angiogenesis in these tumors, we used a double-labeling immunohistochemical technique, simultaneously staining endothelial cells for the expression of CD34 and detecting proliferating cells by using the proliferation markers PCNA and Ki67. This approach facilitated the detection of the relatively few proliferating endothelial cells among the numerous tumor cells (Fig. 1). Counting the CD34-positive microvessels in tumor vascular hot spots revealed relatively uniformly high MVDs in the different tumor types, with glioblastomas and renal cell carcinomas having the highest average MVDs (Fig. 2). Nevertheless, it is noteworthy that MVD counts in all tumors except for prostate carcinomas were lower than the MVD counts for the corresponding normal tissues (brain tissue *versus* glioblastomas, 128%; kidney tissue *versus* renal cell carcinomas, 141%; colon tissue *versus* colon carcinomas, 103%; mammary tissue *versus* mammary carcinomas, 135%; lung tissue *versus* lung carcinomas, 348%; prostate tissue *versus* prostate carcinomas, 68%).

Both PCNA and Ki67 proved to be useful in assessing endothelial cell and tumor cell proliferation. On average, PCNA staining yielded 1.31 ± 0.23 (mean ± SD) times higher values than staining with Ki67, confirming previously reported differences between PCNA and Ki67 (22, 23). When quantitating a PCI reflecting the percentage of capillaries with PCNA-positive endothelial cell nuclei within vascular hot spots, significant differences were detected between the different tumor types (Fig. 2*B*). Glioblastomas (mean ± SD, 9.6 ± 6.1%; median, 8.6%) and renal cell carcinomas (mean ± SD, 9.4 ± 5.2%; median, 8.3%) had significantly higher PCIs than mammary carcinomas (mean ± SD, 5.0 ± 4.8%; median, 3.4%), lung carcinomas (mean ± SD, 2.6 ± 2.5%; median, 2.3%), and prostate carcinomas (mean ± SD, 2.0 ± 1.4%; median, 1.9%; $P < 0.005$). Colon carcinomas had intermediate PCI values of 7.8 ± 5.2% (mean ± SD; median, 6.6%). In contrast, PCI values of all corresponding normal tissues were at the detection limit, with only an occasional PCNA-labeled endothelial cell being detectable (median of all tissues, 0%). The organs of the female reproductive system represent the only organ system with significant physiological angiogenesis (20, 24). We consequently determined PCI values in the cyclic ovarian corpus luteum. The highest PCI values were determined in the growing corpus rubrum (mean ± SD, 40.6 ± 11.2%; median, 37.8%; Fig. 2*B*). PCI values during corpus luteum angiogenesis were significantly higher than the PCI values of all analyzed tumor groups ($P < 0.005$).

When comparing PCI values of individual tumors, a large degree of variation was seen (Fig. 3*A*). PCI values in glioblastomas, renal cell carcinomas, and colon carcinomas varied over a wide range. Some tumors were found to have extremely high PCI values (>20%), whereas others had PCI values that were not higher than those of the groups with low PCI values (mammary, lung, and prostate carcinomas). With few exceptions, PCI values in these tumors were consistently low.

To assess the relative angiogenesis-inducing capacity of different types of tumor cells in relation to their own proliferative capacity, we determined an index of the overall ratio of proliferating tumor cells: proliferating endothelial cells (PTE index; Fig. 2*C*). Glioblastomas

Table 1 *Summary of tumors*

	n	Histology/grading
Glioblastomas	30	All grade IV astrocytomas
Renal cell carcinomas	22	4 grade I and 18 grade II tumors
Colon carcinomas	18	15 grade II and 3 grade III tumors
Mammary carcinomas	24	19 grade II and 5 grade III tumors
Lung carcinomas	15	11 grade II and 4 grade III tumors
Prostate carcinomas	19	3 grade I, 15 grade II, and 1 grade III tumors

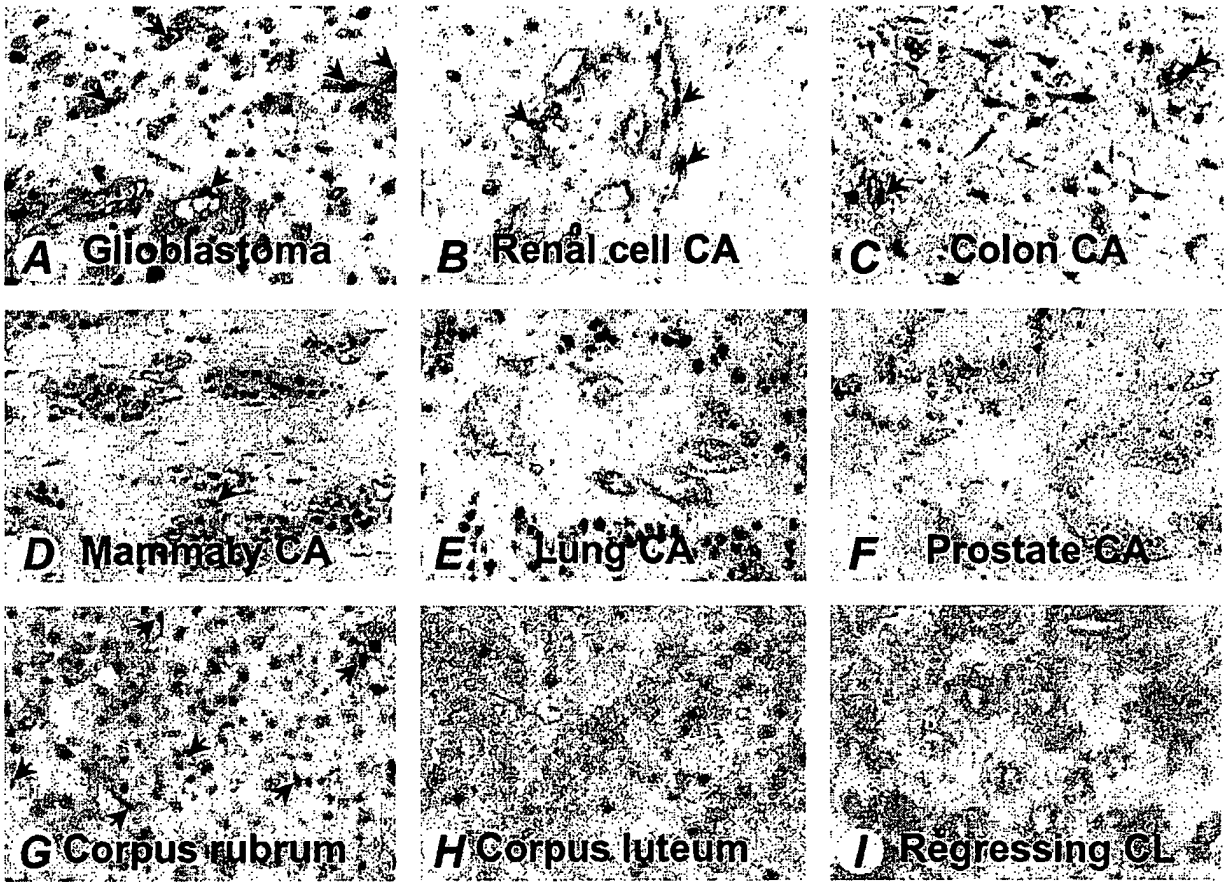

Fig. 1. Histochemical detection of proliferating endothelial cells using a double-labeling technique to stain endothelial cells for CD34 expression (*A–F*) or lectin BS-I binding (*G–I*; *red*) and stain proliferating cells using PCNA as a proliferation marker (*dark blue*). The sections were not counterstained. Thus, all dark nuclei represent PCNA-positive tumor cells and endothelial cells (*arrows*). The double-labeling technique was applied to different types of malignant human tumors. Representative pictures for each tumor type are shown. *A*, glioblastoma; *B*, renal cell carcinoma; *C*, colon carcinoma; *D*, mammary carcinoma; *E*, lung carcinoma; *F*, prostate carcinoma. Endothelial cell proliferation during tumor angiogenesis was compared to the intensity of physiological angiogenesis in the cyclic ovary. *G*, early corpus rubrum, reflecting the most intense phase of ovarian angiogenesis in the first few days after ovulation (days 1–4 after ovulation); *H*, mature midstage corpus luteum with detectable turnover of endothelial cells (days 12–18 after ovulation); *I*, luteolysis with regression of the corpus luteum and all of its vasculature (days 18–24 after ovulation).

and renal cell carcinomas had PTE indices around 10, colon and mammary carcinomas had PTE indices between 20 and 30, and lung and prostate carcinomas had PTE indices between 40 and 70.

Pericyte Coverage of the Neovasculature in Human Tumors. To quantitatively assess the functional status of the tumor neovasculature, we applied a double-labeling immunohistochemical technique to simultaneously stain endothelial cells for CD34 expression and mural cells (pericytes/smooth muscle cells) with an antibody to α-SMA (21). Association of α-SMA with capillary endothelial CD34 expression was interpreted to reflect pericyte staining, whereas α-SMA association with CD34 expression in arteries and veins was interpreted to reflect smooth muscle cell staining (Fig. 4). A MPI was quantitated that reflects the percentage of capillaries associated with α-SMA-positive pericytes. MPIs were determined for all tumor types. Glioblastomas and renal cell carcinomas were identified as the tumor types with the lowest MPI values (glioblastomas, mean ± SD = 12.7 ± 7.9% and median = 9.7%; renal cell carcinomas, mean ± SD = 17.9 ± 7.8% and median = 17.6%; Figs. 2*D* and 3*B*). Mammary carcinomas had the highest MPI values (mean ± SD, 67.3 ± 14.2%; median, 70.4%). Similarly, colon carcinomas also had relatively high MPI values (mean ± SD, 65.4 ± 10.5%; median, 67.7%). Lastly, lung and prostate carcinomas had intermediate MPI values of 40.8 ± 14.5% (mean ± SD; median, 40.6%) and 29.6 ± 9.5% (mean ± SD; median, 29.3%), respectively.

The pericyte coverage data in the different types of malignant tumors were compared with the status of the neovasculature during cyclic ovarian angiogenesis. Angiogenesis in the cyclic ovary is a physiological event with a coordinated program of sprouting angiogenesis and mural cell recruitment. This is reflected by a MPI of 60.2% even in the angiogenic corpus rubrum (Refs. 21; Figs. 2*D* and 4*G*). The MPI of the mature midstage corpus luteum (62.4%) characterizes an overall immature neovasculature that rapidly undergoes regression at the onset of luteolysis.

DISCUSSION

Antiangiogenic targeting of the neovasculature within tumors is considered one of the most promising strategies in the search for novel antineoplastic therapies (5, 6). The concept that tumor vessels can be selectively targeted without affecting the quiescent organ vasculature is based on the fact that the molecular phenotype of immature, angiogenic blood vessels is distinctly different from that of resting blood vessels. A number of molecular determinants of angiogenic endothelial cells have been identified in recent years (4) and are being extensively explored for their suitability to target angiogenic blood vessels. Correspondingly, numerous animal studies have shown that

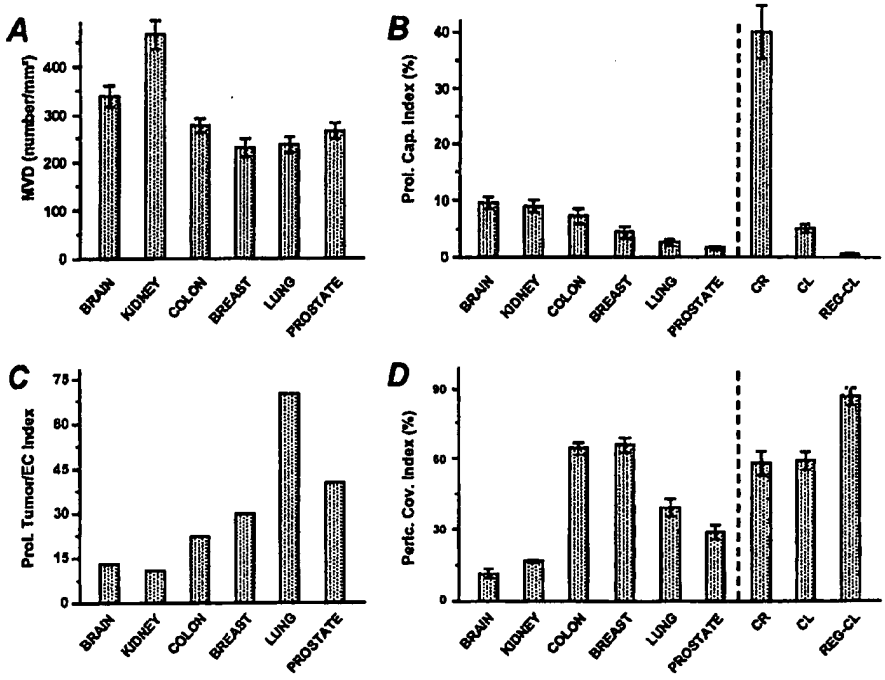

Fig. 2. Quantitative analysis of endothelial cell proliferation and pericyte recruitment during pathological tumor angiogenesis and physiological ovarian angiogenesis. All data are expressed as mean ± SE. *A*, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas have similarly high MVD counts. Glioblastomas and renal cell carcinomas have higher average MVD counts ($P < 0.005$). *B*, a PCI (*Prol. Cap. Index*) was quantitated assessing the percentage of microvessels with proliferating endothelial cells (see "Materials and Methods"). A ranked order of the intensity of angiogenesis in the different types of tumors was determined as follows: glioblastomas > renal cell carcinomas > colon carcinomas > mammary carcinomas > lung carcinomas > prostate carcinomas. PCI values in malignant human tumors were compared with the intensity of angiogenesis in the cyclic bovine corpus luteum [*CR*; early corpus rubrum (angiogenesis)], midstage corpus luteum (*CL*; maturation), and regressing corpus luteum (*REG-CL*; regression). *C*, a quantitative ratio of the total number of proliferating tumor cells in all tumors:the total number of endothelial cells (*Prol. Tumor/EC Index*) was determined for each type of tumor to establish a relative parameter that reflects the per tumor cell angiogenic capacity of the tumors. *D*, a MPI (*Peric. Cov. Index*) was quantitated assessing the percentage of microvessels that are associated with α-SMA-positive pericytes (see "Materials and Methods"). MPI indices of the different types of malignant human tumors were compared with the MPI values of the transient neovasculature during ovarian angiogenesis.

the activated, angiogenic neovasculature can be selectively targeted without affecting the normal organ vasculature (7, 25–27).

Essentially all of the antiangiogenic animal studies have used experimental models with a very high intensity of angiogenesis. These include rapidly growing tumor models such as the Lewis lung carcinoma (7, 25), the rabbit cornea assay with an implanted angiogenic factor (26), or the naturally occurring angiogenic processes in the female reproductive system (27). Contrasting these experimental models with a high intensity of angiogenesis, little is known about the degree of active angiogenesis and the functional status of the vasculature within human tumors. Vessel density counting studies have used panendothelial cell markers that facilitate the quantitation of the number of blood vessels within tumors (17, 18). These studies have demonstrated that high vessel densities in tumors correspond with poor prognosis. However, the use of panendothelial cell markers, such as CD31 or CD34, facilitates the assessment of the vascular status of a tumor but does not give an indication of the tumor's angiogenic status. In fact, recent histomorphological studies indicate that some tumors may be vascularized without significant angiogenesis, probably by using the preexistent organ vasculature (28) or even by forming vascular channels on their own through a nonendothelial cell process designated as vascular mimicry (29). Recent studies have tried to circumvent this problem by using marker molecules that are up-regulated during angiogenesis, such as CD105 (30) and the integrin $\alpha_v\beta_3$ (31).

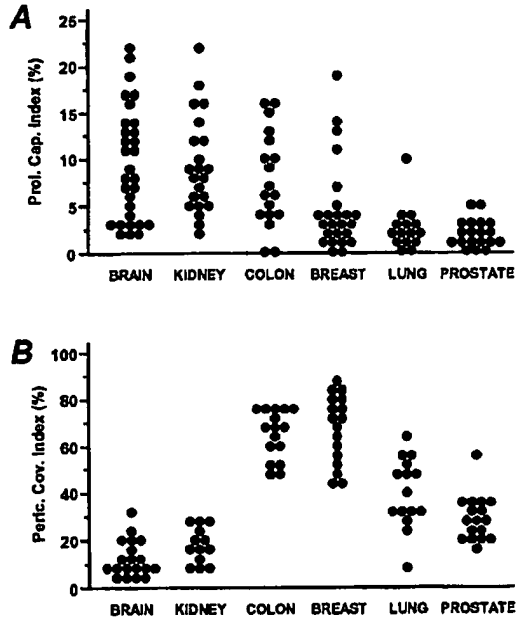

Fig. 3. Endothelial cell proliferation and pericyte recruitment in individual malignant human tumors. *A*, PCIs (*Prol. Cap. Index*) in glioblastomas, renal cell carcinomas, and colon carcinomas vary over a wide range. In contrast, with few exceptions, mammary carcinomas, lung carcinomas, and prostate carcinomas have uniformly low PCI values. *B*, MPIs (*Peric. Cov. Index*) in the different tumor types varied over a much smaller range than the PCIs.

In this study, we have quantitatively assessed the rate of angiogenesis (based on the proliferation of endothelial cells) as well as the functional status of the neovasculature (based on the recruitment of α-SMA-positive pericytes) in six different types of malignant human tumors. The results indicate that there is active angiogenesis in human tumors, albeit at a much lower rate compared with the physiological cyclic angiogenic processes in the ovarian corpus luteum. Furthermore, the varying degrees of pericyte recruitment indicate differences in the functional status of the tumor vasculature in different tumors that may reflect varying degrees of maturation of the tumor vascular bed.

Studies performed as early as 1972 have attempted to establish a procedure for the assessment of the angiogenesis status of tumors (17). To date, however, no standardized scheme is available to reliably assess the angiogenesis status of a given tissue or a tumor. Despite the increasingly recognized distinct phenotypic properties of angiogenic endothelial cells (4, 32), proliferation of endothelial cells may still be considered as the single most reliable parameter to quantitate angiogenesis. Few endo-

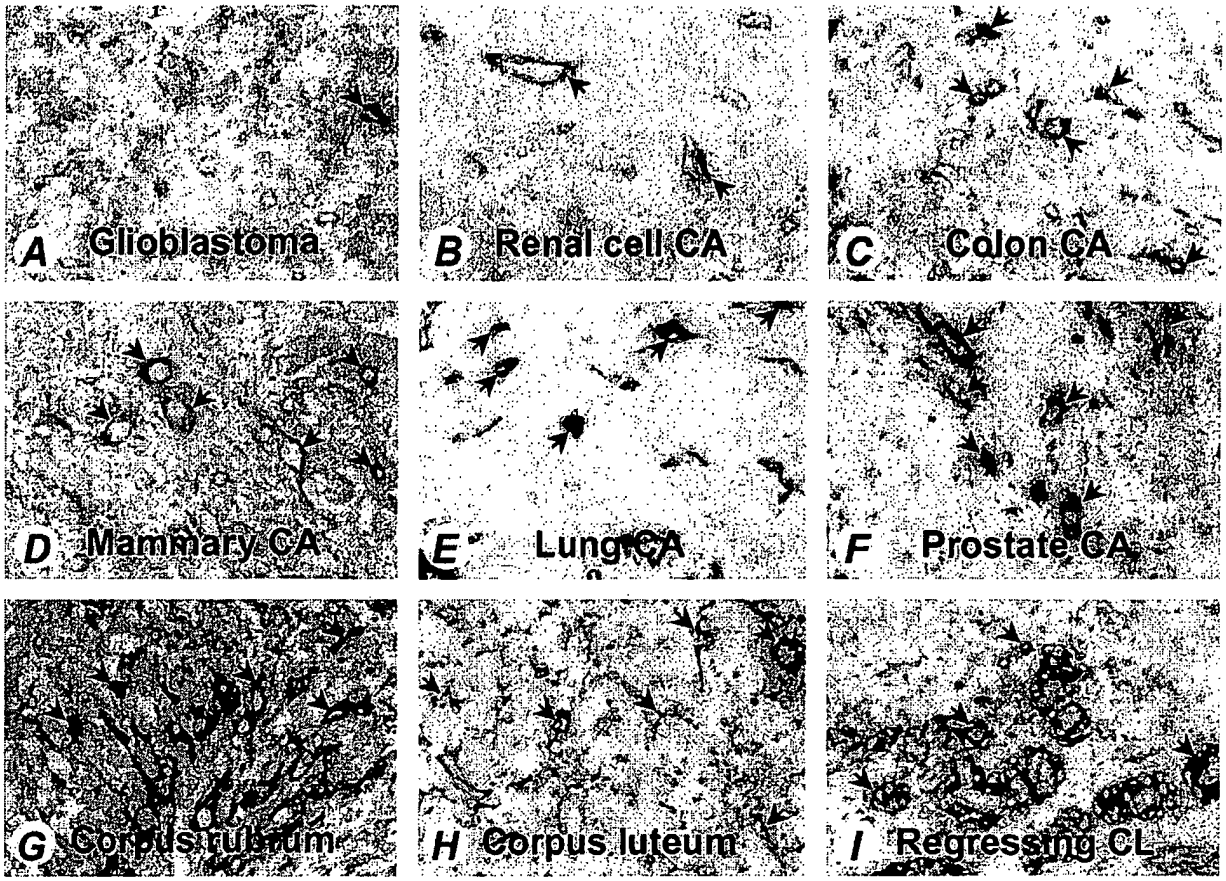

Fig. 4. Histochemical analysis of pericyte recruitment using a double-labeling technique to stain endothelial cells for CD34 expression (*A–F*) or vWF expression (*G–I*; *red*) and stain mural cells (pericytes, smooth muscle cells) for α-SMA expression (*dark blue*). α-SMA staining of microvessels was considered to reflect pericyte staining, whereas α-SMA of larger vessels indicated smooth muscle cell staining. Representative pictures for each tumor type are shown. *A*, glioblastoma; *B*, renal cell carcinoma; *C*, colon carcinoma; *D*, mammary carcinoma; *E*, lung carcinoma; *F*, prostate carcinoma; *G*, early corpus rubrum; *H*, mature midstage corpus luteum; *I*, regressing corpus luteum.

thelial cell proliferation studies in human tumors have been reported (13–16). These studies describe tumor endothelial cell proliferation indices between 0.15% (13) and 9.9% (16). Much of the variation in the literature data may be attributed to differences in methodology. Thus, one of the goals of the present study was to standardize one technique and to comparatively apply this technique to different tumors. When assessing angiogenesis based on endothelial cell proliferation in different types of human tumors, we identified significant differences in the degree of active angiogenesis between different types of tumors as well as within one tumor type. Glioblastomas, renal cell carcinomas, and colon carcinomas were identified as the most angiogenic types of tumors. There was a high degree of variation among the individual tumors, indicative of a low rate of active angiogenesis in a subgroup of these tumors. In contrast to the intense angiogenesis in some types of tumors, lung carcinomas, prostate carcinomas, and most of the mammary carcinomas had relatively low endothelial cell proliferation indices. Nevertheless, these low PCI values (around 2%) are still indicative of active angiogenesis in these tumors. Endothelial cell turnover in the corresponding normal tissues was below the detection limit, with only single PCNA- or Ki67-positive endothelial cells being detectable. A very careful analysis of endothelial cell turnover in normal tissue has identified around 0.1% proliferating endothelial cells in normal tissues (12), which is still lower than the PCI values for prostate and lung carcinomas by a factor of 20. When comparing tumor PCI values to angiogenesis in the cyclic ovary, it became apparent that angiogenesis in human tumors is operative, albeit at a much lower rate than in the corpus luteum. Angiogenesis in the growing corpus luteum in the first few days after ovulation was found to be fourfold to twentyfold more intense than the angiogenesis in the different malignant human tumors (PCI > 40%), corresponding to previous reports on the high intensity of angiogenesis in the female reproductive system (33, 34).

In addition to assessing tumor endothelial cell proliferation, we quantitated the recruitment of mural cells (pericytes, smooth muscle cells) to the tumor neovasculature. The identification of the angiopoietins (35–37) and the phenotype of platelet-derived growth factor-β-deficient mice (inability to recruit pericytes) (38, 39) has focused attention on the molecular mechanisms of blood vessel maturation mediated by the recruitment of pericytes. The mature phenotype of the quiescent organ vasculature in most organs is characterized by an extensive coverage with pericytes that appear to play a role in controlling the quiescent endothelial cell phenotype. Correspondingly, it has long been speculated that the tumor vasculature is characterized by a distinct maturation defect that is at least partially responsible for the irregular, tortuous, and leaky blood vessels found within tumors (40, 41). It was recently shown that androgen ablation therapy of prostate tumors leads to a down-regulation of vascular endothelial growth factor within the tumor, leading selectively to the regression of immature tumor microvessels that were not covered by pericytes (42). In line with these findings, we determined in the present study that only one-third of the vasculature within prostate carcinomas is covered by pericytes, despite the fact that prostate tumors were identified

as not very angiogenic tumors based on the assessment of endothelial cell proliferation.

The degree of pericyte recruitment to the neovasculature in the different tumor types varied significantly. The neovasculature in mammary and colon carcinomas had the highest rate of pericyte coverage, with as many as 70% of all microvessels being in contact with mural cells. In contrast, glioblastomas and renal cell carcinomas had pericyte coverage indices between 10% and 20%, indicating that most microvessels did not establish mural cell contact. These quantitative differences in mural cell recruitment could reflect varying degrees of vessel maturation of the tumor vascular bed. There is good evidence to suggest that pericyte coverage is a correct functional reflection of the degree of microvessel maturation (38, 39, 42, 43). However, it should be noted that pericyte coverage is not the only mechanism of vessel maturation, as indicated by the fact that the quiescent organ vasculature in some organs such as the lungs is not extensively covered by pericytes.

In summary, the present study has demonstrated that malignant human tumors are characterized by varying degrees of angiogenesis and pericyte recruitment. Furthermore, they indicate that the degree of angiogenesis in human tumors varies widely and may be very low in some types of tumors. Despite the fact that little is known about the mechanism of action of most angiogenesis inhibitors, the data suggest that the suitability of tumors for antiangiogenic therapies may differ between different tumor types and even within one type of tumor. Tumors with a low intensity of angiogenesis may not benefit much from antiangiogenic therapies that depend on the rate of endothelial cell proliferation. This stresses the importance that techniques such as those described in this study need to be implemented in clinical practice to assess the angiogenic status of a patient to identify those who will benefit most from antiangiogenic therapy. Furthermore, the data also indicate that the vasculature in most tumors is not very extensively covered by pericytes, which may reflect a functional immaturity of the tumor vascular bed. Not only may pericyte coverage of microvessels control vessel maturation, but it has also been shown to define a plasticity window for blood vessel remodeling (43). Thus, our data provide support for the concept that in addition to antiangiogenic therapies, angioregressive therapies could be developed that are capable of selectively inducing the regression of the immature tumor vasculature with an open plasticity window (42).

ACKNOWLEDGMENTS

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ELSEVIER FULL-TEXT ARTICLE

The angiogenesis inhibitor, endostatin, does not affect murine cutaneous wound healing.

Berger AC, Feldman AL, Gnant MF, Kruger EA, Sim BK, Hewitt S, Figg WD, Alexander HR, Libutti SK.

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BACKGROUND: Endostatin is a potent angiogenesis inhibitor, which is currently being used in Phase I trials as an antitumor agent. The purpose of this study was to determine whether endostatin has an effect on wound healing in a murine model. MATERIALS AND METHODS: The function of endostatin was confirmed using a human microvascular endothelial cell (HMVEC) proliferation assay in which cells are treated for 4 days with growth media plus or minus endostatin. Full-thickness incisions were made on the dorsum of athymic nude mice and closed primarily with skin staples. PVA sponges were implanted in some wounds to determine vascular ingrowth. Subsequently, mice were treated with recombinant human endostatin at 20 mg/kg/day or 50 mg/kg/dose BID versus control for a total of 14 days. On Days 2, 4, 8, 12, and 16, three mice per group had serum samples drawn and were sacrificed. Perpendicular breaking strength (N) was determined using an Instron 5540 tensometer. Wound strength was determined by dividing breaking strength by wound area (N/cm(2)). Vascular density in sponges was determined using CD31 immunohistochemistry. Serum endostatin concentrations were determined using a commercially available ELISA kit. RESULTS: Endostatin caused a significant reduction of endothelial cell proliferation after 4 days compared to media alone (72%, P = 0.031). At all time points tested, there was no statistical difference in the wound-breaking strength between endostatin and control-treated mice at either the low or high dose. Serum endostatin levels were consistently 10-fold higher in endostatin-treated mice than in controls. No differences in vascular density were seen in endostatin versus control-treated mice as determined by CD31 immunohistochemistry of PVA sponges. CONCLUSION: Therapy with human endostatin does not induce a significant decrease in breaking strength of cutaneous wounds in mice.

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Continuous Intravascular Secretion of Endostatin in Mice from Transduced Hematopoietic Stem Cells

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Endostatin, a 20-kDa carboxy-terminal fragment of collagen XVIII, is the leading member of a class of physiologic inhibitors of angiogenesis with potent antitumor activity. Repeated subcutaneous administration of recombinant endostatin in mice led to permanent regression of established tumors to a microscopic dormant state and prompted the initiation of human clinical trials. However, a discrepancy remained unresolved: sustained tumor regression has only been observed with a non-soluble, precipitated form of recombinant endostatin produced in bacteria. To shed light on this question and establish a model of systemic anti-angiogenic gene therapy of cancer that may surmount obstacles in protein production and delivery, we transduced murine hematopoietic stem cells with a retrovirus encoding a secretable form of endostatin. Despite continuous, high-level secretion of endostatin in the vasculature of all transplanted mice, we detected neither inhibition of *in vivo* neoangiogenesis nor antitumor activity. Resolution of this paradox may come from human trials of endostatin now underway.

Key Words: endostatin, anti-angiogenesis, hematopoietic stem cells, retroviral vector, bone marrow transplantation

INTRODUCTION

Although tumor-associated angiogenesis has long been recognized as a key process in cancer progression and metastasis [1,2], the identification of potent angiogenesis inhibitors has remained rather elusive. The discovery of a group of physiological inhibitors that are generated by protease-mediated cleavage of extracellular proteins has raised considerable interest [3]. Among them, endostatin, a 20-kDa carboxy-terminal fragment of collagen XVIII, is especially potent at inhibiting the growth of various murine tumors and their metastases in animal models [4]. Furthermore, endostatin has the remarkable property in cycled therapy of inducing the regression of established tumors to a microscopic dormant state, even after prolonged discontinuation of treatment [5]. These unique properties have ushered the initiation of human clinical trials [6]. However, complete inhibition of tumor growth and tumor regression have only been reported so far with the non-soluble, precipitated form of recombinant endostatin produced in *Escherichia coli* [4,5,7–12]. It was argued that precipitated endostatin injected subcutaneously acts as a depot for slow release of the active protein in the vasculature [4]. Because the non-soluble, precipitated form of endostatin is not suitable for human clinical trials, a soluble version of the human protein was produced in yeast [12]. Although endostatin produced in yeast or mammalian cells was able to inhibit the growth of tumors, it was unable to regress established tumors [8–12] and their residual activity remained difficult to obtain in large quantities [13].

On theoretical grounds, systemic anti-angiogenic gene therapy may overcome these difficulties as well as the requirement for repeated, long-term protein administration by providing sustained therapeutic levels of endostatin in the serum [14]. So far, however, studies aimed at preventing tumor growth by the injection of plasmid DNA (either naked or complexed in liposome formulations) or recombinant adenoviruses encoding either human or murine endostatin have shown only modest inhibition of tumor growth and no tumor regression [15–27]. An important question is whether the observed discrepancy between

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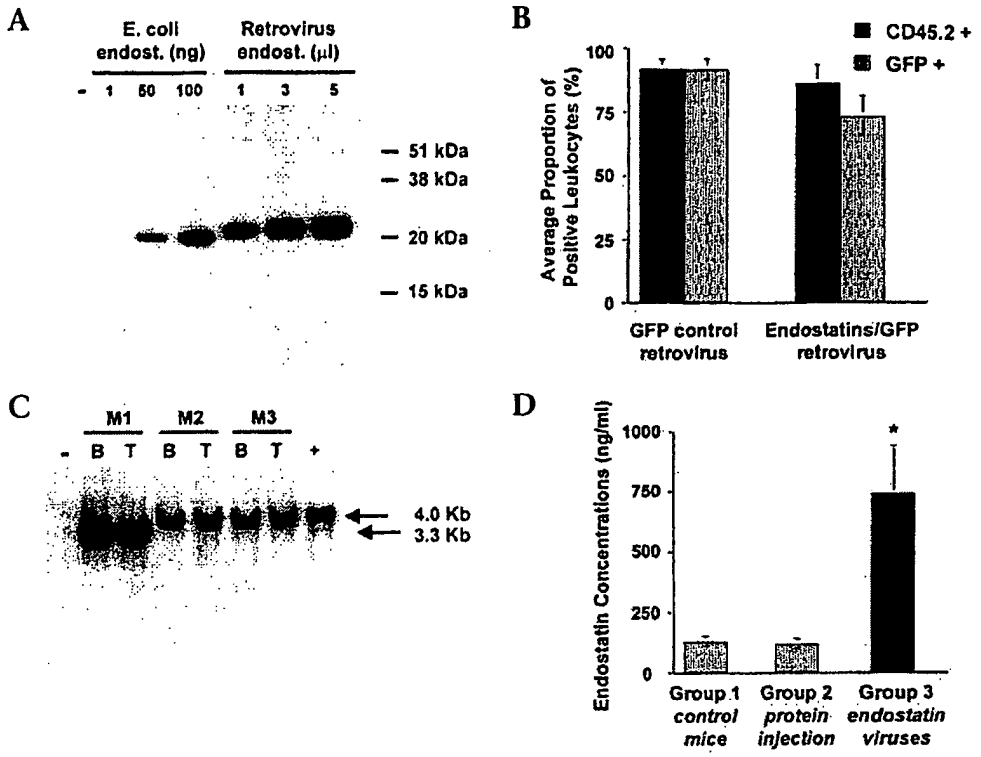

FIG. 1. Analyses of recombinant endostatin production, reconstitution of transplanted mice with donor cells, and proviral stability. (A) Detection of secreted murine endostatin from retrovirally transduced 293T cells by western blot analysis with specific polyclonal antibody. Left lane (–), supernatant from cells transduced with MSCV[GFP] control vector; middle lanes, various quantities of recombinant His-tagged murine endostatin produced in *E. coli*; right lanes, various volumes of heparin Sepharose purified murine endostatin obtained from supernatants of cells transduced with MSCV[FLAG-endostatin/GFP] virus; right margin, molecular size markers. As previously observed, endostatin expressed in mammalian cells migrates slightly slower than bacteria-produced endostatin, presumably because of differences in glycosylation [42]. (B) Quantification of the extent of reconstitution of transplanted mice with donor derived cells, on the basis of expression of the CD45.2 allele, and of the proportion of GFP+ cells among donor-derived cells, 3 months post-transplantation. Blood leukocytes were analyzed by FACS for both CD45.2 expression and GFP fluorescence. Because no statistical difference was observed among mice for MSCV[endostatin/GFP] ($n = 5$) and MSCV[FLAG-endostatin/GFP] viruses ($n = 13$), data were grouped ($n = 18$). For MSCV[GFP] control, $n = 10$. Error bars represent standard deviations. (C) Detection of intact proviruses by Southern blot analysis of bone marrow (B) and thymus (T) genomic DNA from representative mice transplanted 4 months earlier with MSCV[GFP] control (M1) or MSCV[FLAG-endostatin/GFP] (M2-3) transduced marrow. (–), Bone marrow from a non-transplanted mouse; (+), NIH3T3 cell clone with two copies of MSCV[FLAG-endostatin/GFP] provirus. DNA was digested with *Sad*. GFP was used as a probe. (D) Quantification of serum levels of murine endostatin in mice by ELISA. Group 1, control mice transplanted with MSCV[GFP] transduced marrow ($n = 10$); group 2, mice after a 12-day regimen of daily injections of bacteria produced His-tagged murine endostatin (20 mg/kg/day) administered as a purified precipitate as described [4] ($n = 4$); group 3, mice transplanted with marrow transduced with the endostatin expressing viruses ($n = 18$). Because no statistical difference was observed among mice for MSCV[endostatin/GFP] ($n = 5$) and MSCV[FLAG-endostatin/GFP] viruses ($n = 13$), data were grouped ($n = 18$). Error bars represent standard deviations. *$P < 0.001$.

gene transfer and protein studies is due to the production of suboptimal levels of endostatin and/or transient expression following gene transfer.

To investigate whether sustained, high-level release of a soluble form of endostatin can result in tumor regression and to establish a model of systemic gene therapy of cancer through angiogenesis inhibition, we set out to obtain continuous intravascular release of endostatin by retrovirus-mediated gene transfer of a secretable form of murine endostatin into hematopoietic stem cells, followed by engraftment of syngeneic mouse recipients. We constructed two bicistronic vectors based on the murine stem cell virus (MSCV) backbone. These vectors contained cDNAs encoding secretable, FLAG-tagged or non-tagged forms of endostatin. In addition vectors also contained the gene encoding the enhanced green fluorescence protein (GFP) linked in *cis* to the internal ribosomal entry site (IRES) from the encephalomyocarditis virus 3′ of the endostatin cDNA to enable the preselection of retrovirally transduced bone marrow cells. Transplantation of retrovirally transduced, GFP+ fluorescence activated cell sorted (FACS) bone marrow cells into recipient mice resulted in stable, long-term reconstitution of all hematopoietic lineages with GFP+ cells and continuous, high-level expression of endostatin in the serum of all mice. Despite this, neither inhibition of *in vivo* neoangiogenesis nor antitumor

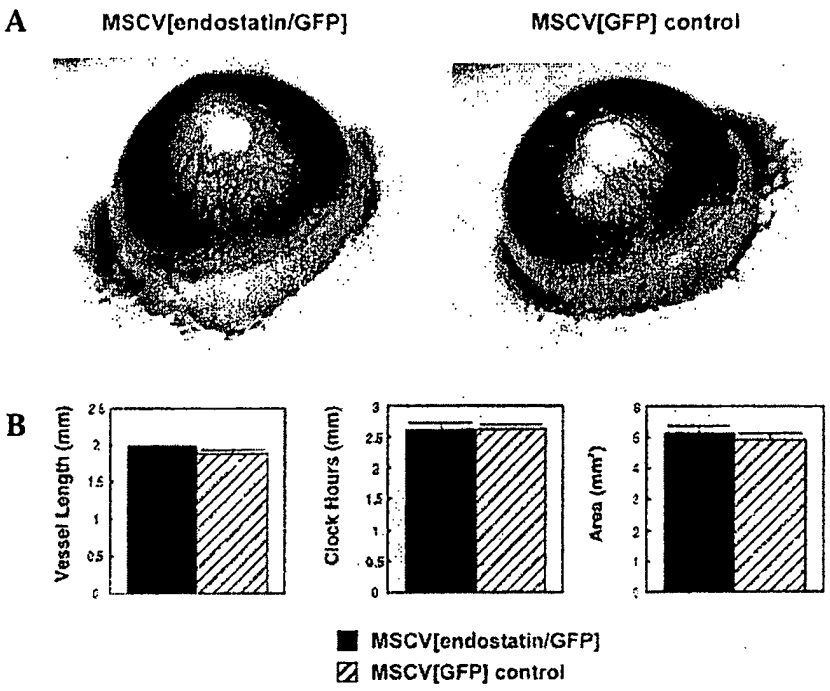

FIG. 2. Lack of systemic inhibition of neoangiogenesis in recipient mice 3 months post-transplantation, as assessed by the corneal micropocket assay with implantation of a FGF-2 pellet. (A) Corneal vascularization achieved in representative recipients of MSCV[endostatin/GFP] (left) or MSCV[GFP] (right) transduced marrow. (B) Quantification of the length (left), clock hours (middle), and area (right) of corneal vessels in mice transplanted with either MSCV[endostatin/GFP] (n = 5) or MSCV[GFP] (n = 4) transduced marrow and subjected to the corneal micropocket assay. Error bars represent standard deviations.

activity was detected. These data demonstrate that sustained intravascular delivery of a soluble form of endostatin does not recapitulate results obtained with non-soluble, bacteria-derived endostatin.

Results

Construction and Characterization of Retroviral Vectors

Originally, endostatin-induced tumor regression was demonstrated with a recombinant protein in which 10 plasmid-encoded amino acids followed by 6 histidine residues (6 × His) were fused to the amino-terminal amino acids of endogenous endostatin [4]. Other studies have used either non-tagged or His– influenza virus hemagglutinin A (HA)- or murine immunoglobulin γ-2A chain (Fc)-tagged endostatin in the same N-terminal position [7–12,28]. We constructed two vectors with or without the FLAG tag, referred to as MSCV[FLAG-endostatin/GFP] and MSCV[endostatin/GFP], respectively, to gather information on both instances. A vector that only expresses GFP, referred to as MSCV[GFP], was used as a gene transfer control.

We generated recombinant retroviruses using the ecotropic packaging cell line BOSC23, with titers greater than 5×10^5 infectious units/ml on NIH3T3 cells. We documented stable proviral transfer by Southern blot analysis of transduced cells (data not shown). To verify the integrity of the secreted proteins and quantify their production, we performed western blot analysis of transduced cell supernatants with a polyclonal antibody specific for murine endostatin (Fig. 1A). Microsequencing of purified secreted endostatin confirmed its authenticity. Proliferation of bovine endothelial cells (BCE) was inhibited by 15–25% in the presence of supernatants of MSCV[FLAG-endostatin/GFP] or MSCV[endostatin/GFP] transduced 293T cells as compared with MSCV[GFP] transduced 293T cells (data not shown).

Preselection and Transplantation of Transduced Bone Marrow

Bone marrow transplantation with transduced stem cells was carried out as follows. We harvested bone marrow from donor C57BL/6 mice, injected intravenously 4 days earlier with 5-fluorouracil (5-FU) to stimulate stem cell division, and exposed it to ecotropic retroviral supernatants. Following infection, we isolated by FACS retrovirally transduced GFP+ cells and injected them into lethally irradiated C57BL/6 recipient mice. We used donor and recipient C57BL/6 mice that are phenotypically distinguishable on the basis of allelic differences at the CD45 locus: donor cells express CD45.2, whereas those of the recipients are CD45.1 [29]. This isolated allelic difference among syngeneic mice does not alter graft tolerance, but enables precise monitoring of the extent of reconstitution with donor-derived cells in transplanted mice [29]. We transplanted a total of 28 mice: 13 with MSCV[FLAG-endostatin/GFP], 5 with MSCV[endostatin/GFP], and 10 with MSCV[GFP] transduced marrow. All mice were kept for a minimum of 3 months post-transplantation and monitored by

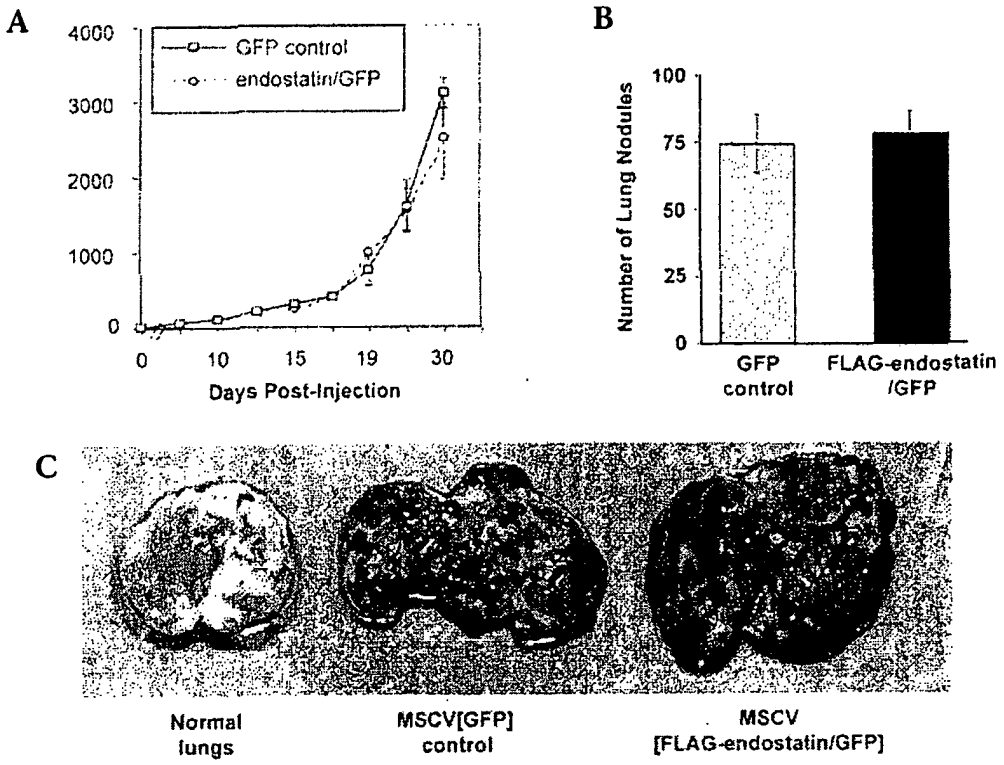

FIG. 3. Quantification of syngenic primary and metastatic tumor growth in transplanted mice with high serum concentrations of endostatin. (A) Growth curves of T241 fibrosarcoma cells injected subcutaneously in syngenic recipients of MSCV[endostatin/GFP] (n = 5) or MSCV[GFP] (n = 4) transduced marrow. Error bars represent standard deviations. (B) Quantification of lung tumor nodules in all mice transplanted with MSCV[FLAG-endostatin/GFP] (n = 13) or MSCV[GFP] (n = 6) transduced marrow, 15 days after intravenous injection of syngenic T241 fibrosarcoma cells. Error bars represent standard deviations. (C) Photographs of lungs from representative mice as described in (B).

quantitative FACS and ELISA analyses of peripheral blood samples. In addition, genomic DNA was isolated from bone marrow and thymus of all mice upon sacrifice for Southern blot analysis. After 3 months post-transplantation, most peripheral blood leukocytes in recipient mice were derived from donor, CD45.2, stem cells and expressed the transferred GFP gene (Fig. 1B). Chromosomal integration of intact provirus in all transplanted mice was confirmed by Southern blot analysis (Fig. 1C).

Sustained, High-Level Expression of Recombinant Endostatin in the Serum of Transplanted Mice

Next, we set out to compare the steady-state serum concentrations of murine endostatin achieved in transplanted mice compared with non-transplanted mice having received 20 mg/kg/day of purified, precipitated recombinant endostatin for 12 consecutive days, a dose that was shown to cause the regression of established primary tumors in mice [4,5]. Serum concentrations were measured with a highly specific polyclonal antibody-based ELISA assay for murine endostatin. As previously observed [30], the average serum levels of recombinant endostatin following the 12-day, high-dose subcutaneous injection regimen were not raised over background levels: 117 ± 16 ng/ml (n = 4) versus 129 ± 16 ng/ml (n = 4), respectively (Fig. 1D). A plausible explanation for the low serum levels achieved by subcutaneous injection of non-soluble endostatin is that the molecules of endostatin, which are slowly released from the depot, rapidly bind to the endothelial cell lining of vessels [28]. In contrast, the production of endostatin by transduced blood and marrow cells of transplanted mice was such that serum endostatin levels rose to 746 ± 197 ng/ml (n = 18; Fig. 1D). No statistical difference was detected between levels achieved with MSCV[FLAG-endostatin/GFP] and MSCV[endostatin/GFP] viruses. Thus, the levels of recombinant endostatin present in the serum of mice transplanted with endostatin transduced marrow was, on average, 7.5-fold greater than the levels detected in the serum of mice following the 12-day injection regimen with 20 mg/kg/day of purified protein (Fig. 1D).

Lack of Inhibition of Neoangiogenesis, Primary Tumor Growth and the Growth of Metastases in Recipients of Endostatin Transduced Bone Marrow

To determine whether inhibition of neoangiogenesis could be detected *in vivo* in transplanted mice that had high steady-state serum levels of endostatin, we carried out a corneal micropocket assay [31]. No inhibition of angiogenesis was observed in mice transplanted with MSCV[endostatin/GFP] transduced marrow (n = 5) compared with control MSCV[GFP] mice (n = 4; Fig. 2). Also, we observed no obvious abnormalities or delay in the healing of tail wounds following peripheral blood isolation in any of the mice throughout the 4-month post-transplantation period.

To investigate whether continuous, high-level, intravascular secretion of murine endostatin would inhibit primary tumor growth in transplanted mice, we injected syngeneic T241 fibrosarcoma cells subcutaneously and measured tumor growth for 30 days. All the mice that were submitted to the corneal micropocket analysis were included in this tumor assay. T241 fibrosarcoma was chosen because it had been reported to be effectively placed in a permanent state of tumor dormancy when tumor-bearing mice were injected subcutaneously with purified, precipitated recombinant endostatin [4,5]. A surprising finding was that no inhibition of tumor growth was observed in recipients of MSCV[endostatin/GFP] (n = 5) transduced marrow as compared with recipients of control MSCV[GFP] transduced marrow (n = 4; Fig. 3A). We next asked whether transplanted mice would be refractory to the development of metastases in an intravenous quantitative metastatic model. We injected recipients of MSCV[FLAG-endostatin/GFP] (n = 13) and MSCV[GFP] (n = 6) transduced marrow with syngeneic T241 fibrosarcoma cells, and 15 days later the lungs were removed and analyzed (Figs. 3B and 3C). No decrease in the number or growth rate of lung tumor nodules was detected (Figs. 3B and 3C).

Although bone marrow engraftment with neo-antigens following lethal irradiation is known to induce a state of tolerance in most instances [32], we analyzed serum samples for the possible presence of neutralizing antibodies. No antibody directed against recombinant endostatin could be detected in the serum of transplanted mice.

Discussion

Here, we have demonstrated that transfer of a retroviral vector encoding a secretable form of murine endostatin into hematopoietic stem cells results in high-level, long-term secretion of the protein in the vasculature of all transplanted mice. Despite this achievement, however, no inhibition in neoangiogenesis or the growth of primary or metastatic tumors was observed. Similiar results were obtained using a NOD/SCID model of human leukemia in the companion paper in this issue [33]. How could these unexpected findings be explained? Serum endostatin concentrations in transplanted mice were 7.5-fold higher than those observed in mice having been submitted to a 12-day regimen of subcutaneous injection of high-dose (20 mg/kg/day) recombinant protein. In our hands, no significant increase in serum concentration could be detected following this regimen of repeated subcutaneous injection of recombinant endostatin. Accordingly, no significant inhibition of tumor growth could be documented ([30] and data not shown). The reasons for this discrepancy with other reports are unknown. The serum levels of endostatin documented in this study were within the range observed in studies using recombinant adenoviral vectors in which an anti-angiogenic and/or anti-tumor effect was reported [23–27]. Thus, it is unlikely that serum concentrations were below therapeutic levels. Furthermore, the integrity of the secreted proteins was verified by western blot analysis and microsequencing, and no neutralizing antibody was detected in the serum of transplanted mice. In addition, the biological activity of recombinant endostatin present in supernatants of transduced cells was verified by its ability to specifically inhibit the proliferation of bovine endothelial cells *in vitro*. Loss of activity by potential degradation of the protein termini is unlikely, as recombinant endostatins lacking N- and/or C-terminal pentapeptide sequences are as active as intact endostatin [34]. It is also unlikely that potential improper folding of the secreted endostatin was to blame, as the most active protocol so far makes use of bacteria-derived recombinant endostatin that precipitates after purification [4]. It is possible that continuous secretion of endostatin does not provide the benefits of intermittent, cycled therapy, for reasons we do not understand. Although much is known with regard to the ability of endostatin to induce endothelial cell apoptosis [35] and its interaction with a variety of extracellular matrix proteins [10] and heparin sulfate [28], much of the molecular details of its mode of action remain unknown. Recombinant endostatins produced in yeast [8,9,12], mammalian cells [10,11,34], or tumor cells following gene transfer [17,18] have shown perceptible activity in mouse tumor models, albeit without evidence of tumor regression. Because the variability in tumor growth following subcutaneous injection of tumor cells is considerable, the resolution of the paradox uncovered by various studies will probably await the results from animal experiments and human clinical trials conducted with large numbers of subjects.

Here, we established that transfer of a retroviral vector encoding a secretable form of a putative antiangiogenic protein into hematopoietic stem cells results in high-level, long-term secretion of the protein in the vasculature of all transplanted mice. This approach should be of substantial value in the identification and assessment of novel angiogenic inhibitors with antitumor properties.

Materials and Methods

Vector construction and cell lines. The mouse procollagen type XVIII α1 cDNA [36] was used as a template to amplify the published sequence of murine endostatin [4] by PCR using two sets of primers encoding the murine IgGκ secretory signal (pSecTag, Invitrogen, Carlsbad, CA), and either did or did not encode the FLAG tag (IBI, New Haven, CT). Integrity of the secretory signal, FLAG tag, and endostatin coding sequence was verified by sequencing both DNA strands. Three vectors were constructed. All vectors were based on the MSCV backbone [37] and contained, 3′ of the endostatin cDNA, the post-transcriptional regulatory element (PRE) from the hepatitis B virus [38] and the IRES element from the encephalomyocarditus virus (Novagen, Madison, WI) fused 5′ to the GFP gene (Clontech, Palo Alto, CA). The amino acid sequence of the secreted forms of endostatin encoded by FLAG-tagged and non-tagged endostatin was NH2-AGDLDTKDDDDKLAHTH and NH2-AAQHTH respectively (the N-terminal residues of murine endostatin are underlined). The amino acid residues 5′ of the non-tagged endostatin sequence represent residues encoded by engineered cloning sites. The third vector, containing all

of the aforementioned elements but lacking an endostatin cDNA, was constructed as a control. T241 fibrosarcoma cells (a gift from Judah Folkman, Children's Hospital, Boston, MA), 293T cells, and the transient packaging cell line BOSC 23 [39] were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with penicillin/streptomycin (Pen/Strep) and 10% heat inactivated (30 minutes at 55°C) fetal calf serum (Hyclone, Logan, UT). Primary bovine capillary endothelial (BCE) cells were isolated and cultured as described [40].

Preparation of purified recombinant murine endostatin from E. coli. The 552-bp PCR fragment encoding murine endostatin was cloned into the PQE.40 expression vector (Qiagen, Valencia, CA) 3' of a leader sequence encoding a 6 × His tag. The N-terminal amino acid sequence of the fusion protein encoded by this vector was NH_2-MRGSHHHHHHGSGGKLAHTH (the N-terminal residues of murine endostatin are underlined). This vector was transformed into the *E. coli* strain MP15 (Qiagen, Valencia, CA). Purification under denaturing conditions was performed according to the manufacturer's instructions (Qiagen, Valencia, CA). After elution from Ni^{2+}-NTA beads, the solution was dialyzed against PBS at 4°C for 72 hours using Spectrum spectra/pore membrane MWCO 6000–8000 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA). Approximately 90% of the purified endostatin precipitated during this step and was collected by centrifugation. The precipitated fraction was resuspended in PBS and its concentration assessed by weighing a desiccated aliquot. Precipitated endostatin was adjusted to a concentration of 3 mg/ml, aliquoted in 300 μl fractions, and used for the *in vivo* experiments.

Generation of recombinant retrovirus stocks. Retroviral construct DNAs were introduced into the transient packaging cell line BOSC 23 [39] using a classical $CaPO_4$ transfection method. Supernatants were collected 48 hours later, filtered through 0.45-μm filters, and either used immediately or frozen at –80°C. Viral titers were determined by exposing 1 × 10^5 NIH3T3 cells to serial dilutions of filtered virus preparations in the presence of 8 μg/ml protamine sulfate, and assessing the proportion of GFP+ cells by flow cytometry 48 hours later. The presence of replication competent retrovirus (RCR) was assessed by the ability to serially transfer viruses conferring G418 resistance to NIH3T3 cells.

Transduction of mouse bone marrow with recombinant retrovirus and assessment of reconstitution with donor cells. Transduction of bone marrow cells was carried out as described [41]. Briefly, bone marrow cells obtained from the hindlimbs of CD45.2 donor mice (The Jackson Laboratory, Bar Harbor, ME), injected 4 days previously with 150 mg/kg 5-fluorouracil (5-FU), were prestimulated for 48 hours in α-medium supplemented with 15% fetal calf serum (Stem Cell Technologies Inc., Vancouver, Canada), 10 ng/ml human IL6, 6 ng/ml murine IL3, and 100 ng/ml murine stem cell factor. All cytokines were bought from PeproTech (Rocky Hill, NJ). Cells were exposed to filtered virus preparations, supplemented with the above cytokines, on Retronectin (Biowhittaker, East Rutherford, NJ) coated tissue culture dishes for 2 days and GFP+ cells were sorted by FACS 48 hours postinfection. Following selection 5 × 10^5 to 1.4 × 10^6 GFP+ bone marrow cells were injected into CD45.1 recipient mice (National Cancer Institute, Bethesda, MD) given 950cGy of whole body irradiation. Blood samples (50 μl) were depleted of erythrocytes by incubation for 10 minutes on ice in the presence of 4 volumes of 1 mol/L NH_4Cl solution and stained with 1 μg/ml phycoerythrin-labeled anti-CD45.2 antibody (Pharmingen, San Diego, CA). Cells were washed with PBS containing 2 μg/ml propidium iodide (PI; Sigma, St. Louis, MO) to distinguish dead cells and concurrently analyzed for GFP and phycoerythrin mediated fluorescence on a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

Southern blot analysis. Genomic DNA was isolated from primary bone marrow and thymus using DNAzol (Invitrogen, Carlsbad, CA). Genomic DNA (10 μg) was digested with *SacI*, electrophoresed through a 0.8% agarose TAE gel, and transferred to nitrocellulose overnight by capillary action. Membranes were probed with the GFP cDNA labeled with α-^{32}P by random oligo priming. Blots were exposed to Kodak XAR film at –70°C for 1–2 days.

Endothelial proliferation assay. Bovine capillary endothelial (BCE) cells were isolated and cultured as described [40]. Cells growing in gelatinized six-well plates were dispersed in 0.05% trypsin solution and resuspended in medium containing 5% bovine calf serum (BCS). After trypsinization, approximately 1 × 10^4 cells in 0.5 ml medium were added to each gelatinized well of 24-well plates and incubated at 37°C for 30 minutes. Samples of endostatin were analyzed in triplicate. Endostatin samples were added to each well and after 30 minutes of incubation, FGF2 (Peprotech) was added to a final concentration of 1 ng/ml. After 72 hours, cells were trypsinized and counted with a Coulter counter (Coulter Electronics Ltd., Fullerton, CA).

Mouse corneal micropocket assay. The corneal micropocket assay was performed as described [31]. Briefly, micropockets were created in the corneas of transplanted mice with a cataract knife. A sucrose aluminum sulfate, hydron polymer pellet coated with 80 ng FGF2 was implanted into each pocket and 5 days later corneal neovascularization was examined with a slit lamp biomicroscope. Vessel length and clock hours of circumferential neovascularization were measured as described [31].

Quantification of tumor growth in recipient mice. For primary tumor studies, 1 million syngeneic T241 fibrosarcoma cells were resuspended in 0.1 ml PBS and subcutaneously injected into each bone marrow recipient at the dorsal midline. Tumor volumes were calculated according to the following formula: width^2 × length × 0.52 [4,5]. For metastases assays, 5 × 10^5 non-transduced T241 fibrosarcoma cells were intravenously injected into each transplanted recipient mouse in a total volume of 0.1 ml PBS.

Quantification of recombinant endostatin in the serum of recipient mice. Quantification of murine endostatin in mouse serum was carried out using the Accucyte ELISA kit according to the manufacturer's instructions (Cytimmune, College Park, MD).

Purification and analysis of secreted recombinant endostatin. Serum-free supernatants from endostatin and control vector transduced 293T cells were harvested and used for purification of endostatin with heparin-Sepharose (Pharmacia, Piscataway, NJ). Approximately 2 ml supernatant was mixed with 200 μl of 20% heparin-Sepharose in TNE buffer and rotated overnight at 4°C. Beads were washed five times with TNE buffer and the bound materials were released by addition of sample buffer containing SDS and DTT, and boiling for 4 minutes. Samples were run on SDS-PAGE gels and transferred to nitrocellulose membrane. Membranes were incubated with a polyclonal rabbit-anti-mouse endostatin antibody (gift of Judah Folkman, Childrens Hospital, Boston, MA) diluted 1:2000 in PBS. Membranes were then incubated with donkey-anti-rabbit IgG HRP-labeled secondary antibody (Amersham, Piscataway, NJ) diluted 1:2000 in PBS. Proteins were visualized using the ECL+ system (Amersham, Piscataway, NJ). To prepare endostatin samples for microsequencing, supernatants from endostatin-transduced 293T cells were loaded onto a heparin-Sepharose 6B column equilibrated with TNE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl). The column was washed five times with 10 ml TNE and endostatin was eluted with 10 ml of 0.75 M NaCl in 10 mM Tris and 1 mM EDTA buffer. The sample was dialyzed against water at 4°C overnight, lyophilized, and resuspended in water. Endostatin was further purified by size-fractionation chromatography using Sepharose 12 coupled to FPLC, pre-equilibrated with 20 mM Tris, pH 8.0. Fractions containing endostatin were concentrated and desalted with Centricons (Millipore, 10 kDa molecular weight cutoff). N-terminal microsequencing was performed for both tagged and untagged endostatin by the Massachusetts Institute of Technology Biopolymers Laboratory.

Analysis of serum for the presence of neutralizing antibodies to murine endostatin. We coated 96-well ELISA plates (Co-Star, Acton, MA) with either native recombinant murine endostatin (Accucyte ELISA kit) or FLAG peptide overnight at 4°C. Plates were washed three times with wash buffer (PBS + 0.05% Tween 20) and blocked for 2 hours at room temperature with 0.1% BSA in 0.9% NaCl and 2% sucrose solution. Wells were incubated, for 45 minutes, with dilutions of serum from control, non-transplanted mice, or mice transplanted with MSCV[FLAG/endostatin/GFP] or MSCV[endostatin/GFP] transduced bone marrow. Plates were washed and incubated with goat-anti-mouse IgG [Fc-specific] horseradish peroxidase conjugated polyclonal antibody (Oncogene Research Products, Cambridge, MA) diluted to 1 μg/ml in PBS + 0.2% BSA for 45 minutes at room temperature. After washing, plates were incubated with tetramethylbenzadine liquid substrate (Sigma) for 45 minutes and stopping reagent was added. Optical density was determined using an optical plate reader at 450 nm (Tecan, Durham, NC).

Statistical analyses. All statistical analyses were performed with the nonparametric Mann-Whitney U-test.

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